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Award Number: W81XWH-09-1-0048

TITLE: A New Approach Targeting Myeloid-Derived Suppressor Cells for Therapy of Mammary Carcinoma

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REPORT DATE: March 2011

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

☒ Approved for public release; distribution unlimited

☐

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
31-MAR-2011	Annual	1 Mar 2010 - 28 Feb 2011

4. TITLE AND SUBTITLE
A New Approach Targeting Myeloid-Derived Suppressor Cells for the Therapy of Mammary Carcinoma

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-09-1-0048

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Miami

Miami, FL 333136

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research
And Material Command Fort Detrick, MD 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES
N/A

14. ABSTRACT

The current therapies against Breast Cancer are limited by the intrinsic genetic instability of the neoplastic cells that facilitates the instauration of multidrug resistance mechanisms and promotes tumor escape mechanisms by altering the surface molecules recognized by active and passive immunotherapies.
To overcome these limitations with the current project we are seeking to target by the use of doxorubicin conjugated RNA aptamer not the evasive neoplastic cells but host derived tumor associated Myeloid derived suppressor cells that compose up to 50% of the tumor mass. These cells induce T cell anergy and suppression, promote and stabilize tumor vasculature and metastatic activity. We successfully generated two aptamers recognizing molecules expressed on these cells and we developed a method to conjugate and purify doxorubicin conjugated aptamer with the intention to use them in vivo.

15. SUBJECT TERMS
MDSC, Cd11b aptamer, IL4Ra, mammary carcinoma therapy

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	13	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

	Page
**Introduction……..…….. **	**4**
**Body…….. **	**4**
**Key Research Accomplishments…….…….. **	**4**
**Reportable Outcomes…… **	**4**
**Conclusion…… **	**11**
**References…… **	**12**
**Appendices…… **	**13**

The current therapies against Breast Cancer are limited by the intrinsic genetic instability of the neoplastic cells that facilitates the instauration of multidrug resistance mechanisms and promotes tumor escape mechanisms by altering the surface molecules recognized by active and passive immunotherapies[1-5].

To overcome these limitations with the current project we are seeking to target by the use of doxorubicin conjugated RNA aptamer not the evasive neoplastic cells but host derived tumor associated Myeloid derived suppressor cells that compose up to 50% of the tumor mass[6-9]. These cells induce T cell anergy and suppression, promote and stabilize tumor vasculature and metastatic activity[9-11].

Introduction, milestones and accomplishment:

During the first year of this award we establish an protocol for SELEX[12-14] optimized for the selection of aptamer against "difficult", negatively charged protein such CD124 (IL4Ralpha). We also isolated an aptamer specific for IL4Ralpha with a biological activity in vitro (apoptosis of immortalized MDSC cell line) and in vivo (reduction of tumor growth) although data were still preliminary. Difficulties related to the change in the postdoctoral fellow in charge of the experiments and the necessity to optimize the SELEX protocol however, did not allow us to select the anti CD11b aptamer. However, an unexpected question was open: how the anti-IL4Ralpha aptamer inhibit tumor growth and MDSC survival.

In the second year of this award we planned 1) to conjugate the aptamer with doxorubicin, and 2) to characterize the in vivo function of the generated aptamers. As discussed later, aptamer against CD11b have been generated, the protocol for the conjugation and purification of doxorubicin with the aptamer established, and the condition for using the new aptamer-doxorubicin determined. Moreover, we confirmed the antitumor efficacy of the anti-IL4Ralpha aptamer and we characterized the mechanisms by which the anti-IL4Ralpha aptamer inhibit tumor growth and promote tumor immunity.

Tasks 1a and 1b required the selection of aptamers against IL4Ralpha and CD11b as well as aptamers isolated by cell selex. These tasks were partially terminated in year one with the isolation of IL4Ralpha aptamer and the initiation of the cell selex. During this second year we successfully isolate aptamers against CD11b and progress further with the cell selex. However, additional cycles are still necessary in the cell selex procedure to obtain MDSCs specific aptamers.

Task 2a, 2b and 2c required to establish the conditions to conjugate the aptamers to the doxorubicin, the methods for their purification and the evaluation of their activity in vitro and in vivo. Moreover, since the anti-IL4Ra aptamer, generated during the first year, showed a biological activity even if not conjugated to the doxorubicin, an additional unexpected task (**task 2d)** needed to be performed. **Task 2a** (aptamer production and conjugation with doxorubicin) has been accomplished. **Task 2b** (determination of the aptamer specificity) was accomplished. **Task 2c** required the in vivo administration of the aptamer conjugated with the doxorubicin to determine their antitumor activity. This task has been initiated but repetition need to be performed in order to confirm the preliminary results and determine if synergy exist when multiple aptamers are used to carry doxorubicin.

Task 2d was not originally contemplated because it derived from the unexpected observation that the anti-IL4Ra aptamer has per se a biological activity. As discussed later, not only we proved that the anti-IL4Ra can block the downstream signaling of this receptor but, also, that activation of this pathway is fundamental for MDSCs survival.

Results:

<u>A new anti-CD11b aptamer</u>

Generation of aptamer against CD11b:

The optimized SELEX protocol established in the first year, has been used to generate aptamers against CD11b. One unforeseen problem had to be solved, the recombinant murine protein, available from abcam when the grant was submitted, became commercially unavailable. Considering 1) the analogy between human and mouse protein and 2) the fact that most antibodies against CD11b crossreact within the two species, we decided to use the still commercially available recombinant human protein. Briefly, rhCD11b was conjugated against epoxy magnetic beads and used to isolate the ligands from the RNA pool. After 5 cycles of binding and amplification the selected, cy3 labelled RNA pool showed, compared to the initial pool, an increase binding on the beads conjugated with the rhCD11b protein (fig1A) as revealed by the higher MFI detected by flow cytometry. As

expected, the MFI further increased at cycle 10. To determine the Kd, in a parallel experiment, different concentrations of Cy3 conjugated aptamers from cycle 5 or 10 were incubated with a fixed amount of CD11b conjugated beads. A four parameters logistic curve was interpolated with the experimental data and reveal that the aptamer pool at cycle 5 has an apparent Kd of 108nM while at cycle 10 the Kd is 16nM. Similar results have been obtained when aptamer binding to the CD11b-beads was revealed by RT-PCR suggesting that Cy3 labeling does not affect aptamer binding.

To determine whether the selected aptamer could bind not only the recombinant human CD11b present on the beads but, also the native murine CD11b, the $CD11b^+$ murine cell line MSC2, was incubated for 15' minutes with Cy3 labeled aptamer either from the cycle 5 or the cycle 10. As negative control, the 4T1 CD11b negative tumor cell line was used. As shown in figure 1b the selected aptamers can recognize the $CD11b^+$MSC-2 while the CD11b negative cell line is not recognized strongly suggesting that the aptamers raised against hCd11b crossreact with the murine counterpart.

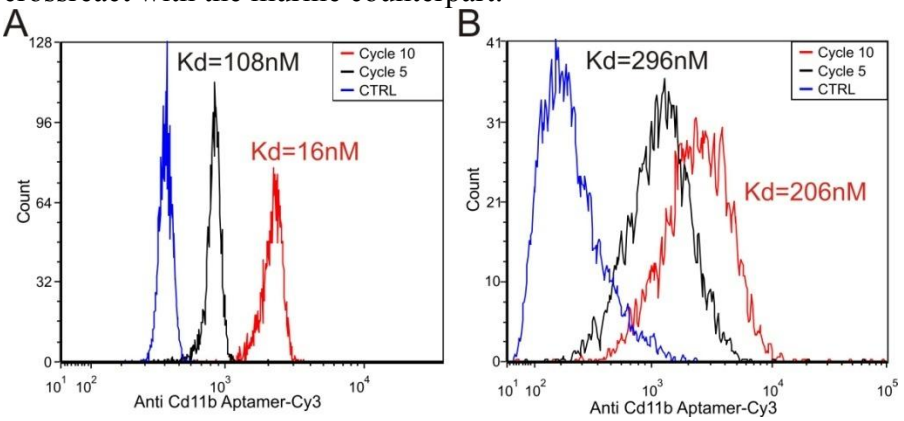

Figure 1: anti-CD11b aptamers recognize both human and murine CD11b. *Aptamers were selected by SELEX using epoxybeads conjugated with rhCD11b. Pool of aptamers from cycle 5 (black line) and 10 (red line) were labeled with Cy3 and incubated either (A) with epoxybeads conjugated with human CD11b or (B) with $CD11b^+$ MSC2 cell line (a murine MDSCs cell line). As negative controls (blue line) irrelevant beads (A) or CD11b- cell lines (B) were used. Kd calculated in a parallel experiment is reported.*

CD11b aptamer: Sequence analysis

Anti CD11b Aptamers from Cycle 10 were retrotranscribed and amplified with primers containing BamH1 and EcoR1 tail. The products were digested with the two restriction enzymes and cloned into pcDNA3.1. Although the use of PCR based cloning systems (i.e. TA cloning) would have been preferable because of the certainly that the 40 random nucleotide would not be cutted, this cloning methods became necessary because of the extremely low yield (associated with the small PCR fragment size) that was obtained with the commercially available TA kit. Some considerations however make this strategy a feasible one: 1) The low provability (40 random nucleotide sequence per aptamer X $(1/4)^6$= 40/4096= 1%) that an aptamer is cutted by the each enzyme and even lower that both cut within the random region of aptamer (0.01%) make quite unlikely that the selected aptamer are cutted. 2) even if this happen, the absence of one or both flanking site in the cloned aptamers should reveal this possibility. In any case, we are evaluating other system based on the protection of 40 random nucleotides but not on the flanking regions from the endunuclease.

The individual clones derived from the pool were sequenced and data analyzed by clustal W and by CARNAC software. Clustal W[15,16] and cladogram analysis reveal the presence of 1 main "family" of closely related aptamers (Fig2). Compare to what we observed for the anti-IL4Ra aptamers (please see report year 1), this family showed an higher complexity and "converged" domain are difficult to be foud just looking at the linear sequence. Since the binding of the aptamers to their ligand is mainly dependent on the secondary and tertiary structure, we evaluate the sequences using a software (CARNAC)[17,18] that compares the RNA considering its secondary structure. Interestingly, this analysis revealed the presence of "conserved" loops in the secondary structure despite the absence of sequence similarity (data not shown).

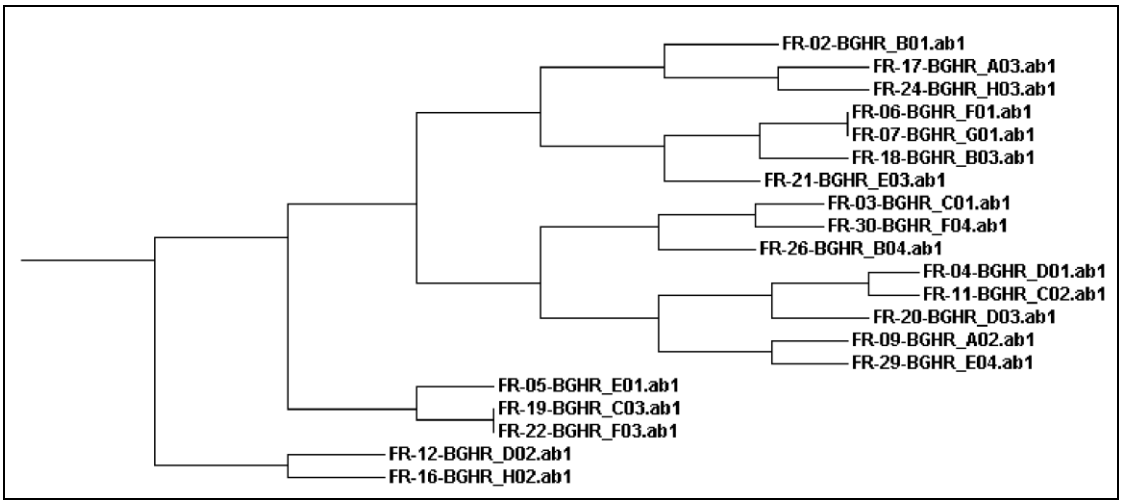

Figure 2: sequence analysis of the anti-CD11b aptamer pool: *Aptamers form the cycle 10 of SELEX were cloned and sequenced. Sequences were analyzed in clustal W giving a weight of 25 for "GAP" opening and 0.05 for gap extension.*

We are currently measuring the affinity, of each clone toward the human CD11b and murine CD11b to choose the one with the lowest Kd for subsequent analysis.

The Anti-IL4Ra aptamer inhibit tumor progression by depleting tumor associated MDSCs.

During the first year we isolate a new aptamer specific for IL4Ra. This aptamer was able to recognize MSC-2 line as well as MDSC isolated from tumor bearing mice. Surprisingly, the anti-IL4Ralpha aptamer seemed to have also a biological function as indicated by the reduction of tumor progression when was administered to tumor bearing mice. These results were unexpected and intriguing and, thus, required to be confirmed and better explained. To this aim we repeat the experiments using either WT or IL4Ra-/- Balb/c mice bearing the 4T1 mammary carcinoma.

Briefly, Balb/c or IL4Ralpha-/- mice were injected sc with the mammary carcinoma or irrelevant aptamers were injected iv every other days tumor progression was monitored. After 20 days all mice were sacrificed, tumor weighted and analyzed by immune fluorescence for the presence of MDSC, and T cells.

As shown in fig.3, anti-IL4Ra significantly delay tumor progression in balb/c mice (fig3a) while no effect was observed when IL4Ra-/- were used (fig3b) or if irrelevant aptamers were injected. This reduction in tumor growth correlated with an higher infiltration of CD3+ T cells (fig.3c-d). This effect could be either indirect, an action on other cells (i.e. MDSC) or direct (a binding of the aptamer to T cells) that, in some particular condition, can express this receptor. To determine which of the two possibilities was more plausible, we performed a set of experiments aimed to identify which cells the aptamers was binding in vivo. To this aim, 4T1 tumor bearing mice were injected with Cy3 conjugated anti-IL4Ra aptamer when tumor reached 0.5 cm of diameter.

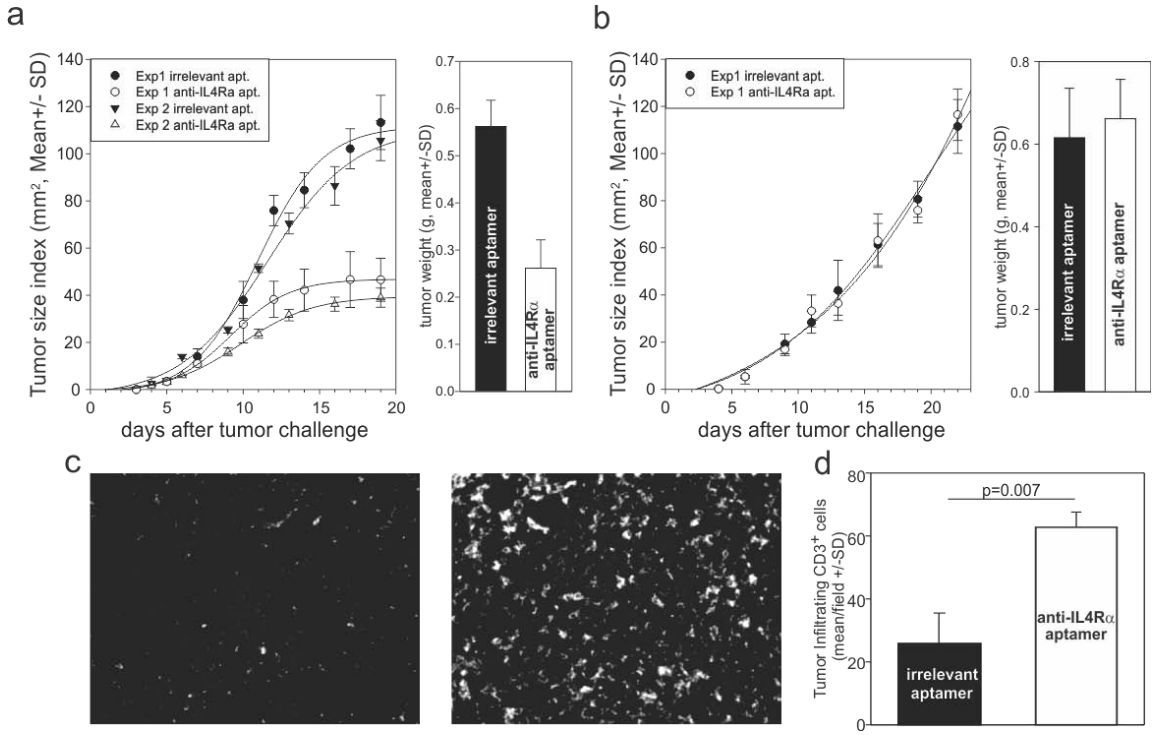

Figure 3: *Anti-IL4Ra aptamer or irrelevant aptamer were injected every other day in Balb/c (A) or in IL4Ralpha-/- (B) mice challenged with 4T1 mammary carcinoma 3 days before initiating the treatment. Tumor progression was evaluated with a caliper and expressed as product of the main diameter and the perpendicular one. C) tumor was removed and weight on day 20. Specimen were also fixed and the number of tumor infiltrating CD3+ cells evaluated by immune fluorescence microscopy (C, D).*

Two hours later mice were sacrificed, spleen, tumor, lung and liver removed, stained with a cocktail of antibodies and analyzed by FACS. As shown in figure 4, aptamer positive cells are found mainly in the tumor of WT mice while no significant accumulation is found on IL4Ra-/- mice (fig.4a-b). Multicolor flow cytometry analysis (fig.4c) shows that the anti-IL4Ra aptamer once injected in vivo binds mostly MDSCs and tumor associated macrophage as determine by the expression of CD11b, Gr1 and F4/80 in aptamer positive cells (fig4c upper panels). No signal is found on B (CD19+ cells) and T cells (CD3+T cells).

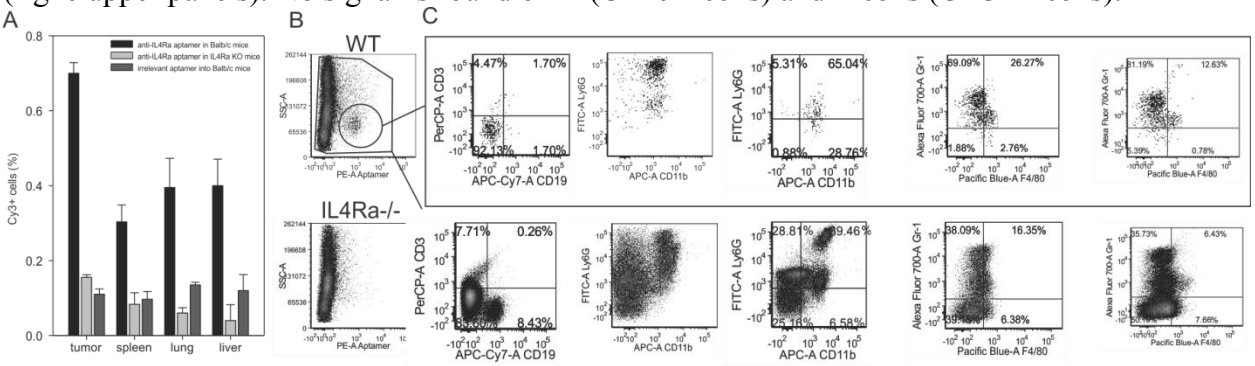

Figure 4: *Cy3 conjugated IL4Ra specific or irrelevant aptamer was injected iv into balb/c or Il4Ra-/- mice bearing a 4T1 tumor (0.5 cm diameter). 2 hours later, mice were sacrificed and the indicated organs (A) were mechanically and enzymatically disrupted into single cells suspension, stained with the indicated antibodies (B, C) and analyzed by FACs. A) The percentage of Cy3+ (aptamer+) cells is reported. B) An example of a plot data from the tumor is reported for the WT Balb/c (upper panel) or IL4Ra-/-(lower panel). C) surface marker expression of the cy3+ cells (upper panels) or tumor mass (lower panels) in the tumor of balb/c mice.*

Thus, these data suggest that anti-IL4Ra antitumor activity is most likely due to an indirect effect of the aptamer on the myeloid population associated with the tumor.

To evaluate the effect on the tumor associated MDSCs and TAM of the cronic administration of anti-IL4Ra aptamers, mice were challenged with the 4T1 mammary carcinoma and treated either with the irrelevant aptamer or with the aptamer specific for IL4Ra. After 20 days mice were sacrificed, tumor removed, formalin fixed and the infiltration of MDSCs was determined by Immune fluorescence.

As shown in figure 5, a significant lower concentration of MDSCs (CD11b+Gr1+cells) and TAM (CD11b+Gr1-) was found in mice treated with the aptamer. This reduction correlated with an higher infiltration of CD8+T cells.

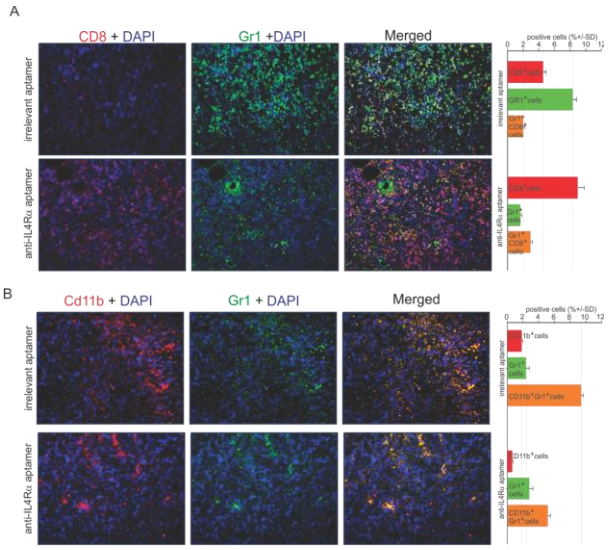

Figure 5: *Cd11b, Gr1 and CD8 immune fluorescence analysis of 4T1 tumor from mice treated with the IL4Ralpha aptamer or with an irrelevant aptamer.*

Taken together, these data seem to indicate that the reduction of tumor growth in anti-IL4Ra aptamer treated mice, is mediated by reduction of MDSCs and TAM that allow a higher infiltration of CD8+ T cells.

The anti-IL4Ra aptamer induced MDSCs apoptosis.

To better understand the effect of anti-IL4Ra aptamer on MDSCs, we decided to use two in vitro models: the MSC-2 cell line and MDSCs isolated from tumor bearing mice.

Briefly, MSC2 were incubated with the aptamer specific for IL4Ra or an irrelevant aptamer as control. After 2 days, culture were harvested and stained with 7AAD and annexin V to evaluate whether an increase in apoptosis was observed. As shown in figure 6a this was the case, A significant increase in MSC-2 apoptosis and a concomitant reduction of viable cells harvested was observed in the well treated with anti-IL4Ra aptamer.

To confirm these findings in a more relevant system, CD11b+ cells were isolated from the spleen of 4T1 tumor bearing mice. MDSC were incubated with the anti-IL4Ra aptamer for 1, 2, 3 or 4 days and viability was evaluated as described above. As shown in fig.6b, an increased mortality in MDSCs was observed.

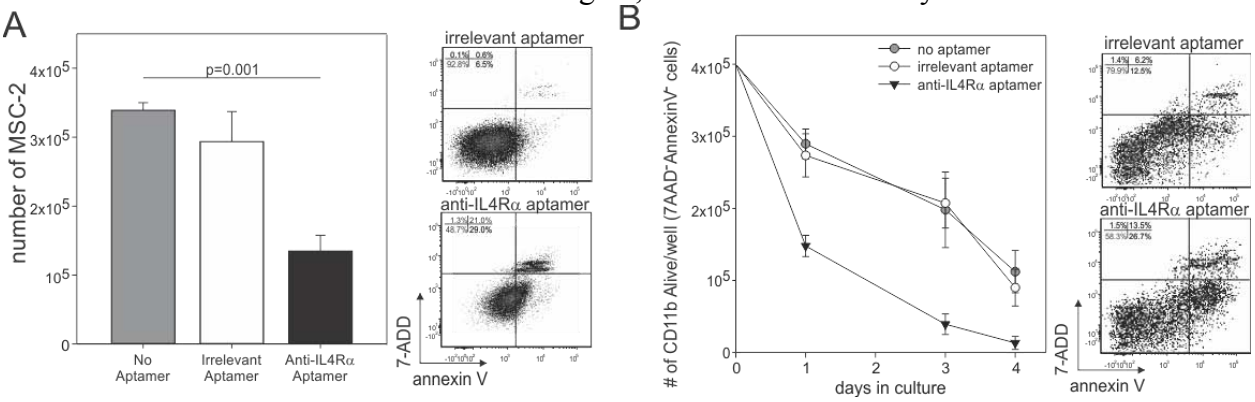

Figure 6: *A) MSC-2 were cultured for 48h alone, with an irrelevant aptamer or with the anti-IL4Ra aptamer. Cells were stained with 7AAD and anti-annexin V and analyzed by FACS. B) CD11b$^+$ cells were magnetically isolated from 4T1 tumor bearing mice, and cultured in media alone, in the presence of the IL4Ra aspecific aptamer or of an irrelevant aptamer as control. Cell viability was evaluated by FACS at day 1, 2, 3 or 4.*

Taken together, these data indicate a pro-apoptotic function on MDSCs of the IL4R alpha aptamer.

The anti-IL4Ra aptamer block the signaling downstream the receptor that is necessary for MDCS survival.

Different mechanisms can be implicated in the aptamer induced apoptosis of MDSCs for example the aptamer could: 1) activate of the proapoptotic signal through the protein kinase R (PKR) that is activate by double strand RNA[19-21], 2) engage TLR3 activating a proapoptotic signaling[22-25], or 3) inhibit some signaling necessary for MDSCs survival. Preliminary experiments using inhibitors of PKR or activators of TLR3 seem to exclude the engagement of these pathways (data not shown). Thus, we evaluate whether the aptamer could block IL4Ra signaling and if this pathway was necessary for MDSCs survival.

To this aim, $CD11b^+$ cells were isolated from the spleen of mice bearing the 4T1 mammary carcinoma and stimulated with IL13 in the presence of anti-IL4Ra aptamer or an irrelevant aptamer as control. Since IL4Ra engagement induces STAT6 phosphorylation{Terabe, 2004 #15816;Wurster, 2000 #15824}, 2h later, the cells were labeled with antibodies against phosphorylated STAT6 (pSTAT6) or with the isotype control. As expected, IL13 induce the phosphorylation of STAT6 (fig7a).While the addition of the irrelevant aptamer do not have any effect on STAT6, the addition of the IL4Ra specific aptamer significantly reduced STAT6 phosphorilation. Although this finding is important, the low increase in STAT6 phosphorilation upon IL13 addition could raise some doubt on these results. The reason for this low increase can be due to the cellular stress induced by CD11b purification or by high basal level of pSTAT6 in these cells. In order to confirm these data in a more controlled environment we decided to use the MSC-2 cell line (fig7b). MSC2 were plated in a 24 well plate for 24 hours to allow them to recover from the use of trypsin. IL-13 was added alone, with the anti-IL4Ra aptamer or an irrelevant aptamer as control. 2 hours later, STAT6 phosphorilation was evaluated by FACS. As observed for the freshly isolated CD11b, the IL4Ra specific aptamer significantly decrease IL13 dependent STAT6 phosphorilation. No activity is observed when the irrelevant aptamer is used.

Taken together these data indicate that the anti-IL4Ra aptamer is a blocking aptamer that prevent IL4Ra signaling. It is important to note that this is one of the first examples of an aptamer with this property. However, these data still do not indicate if this is the cause of MDSCs apoptosis. Indeed it has never been reported that the absence of STAT6 signaling induce MDSCS death.

To determine whether IL13 and or IL4 (the ligands of IL4Ra) promote MDSCs survival we adopted 2 strategies: 1) we used neutralizing antibodies on MSC-2 culture and we evaluate Cell survival, 2) we took advantage of the high mortality seen in freshly isolated CD11b to determine whether IL13 addition can promote their survival.

In the first strategy MSC-2 were plated in 24 well plates for 4 days in the presence of neutralizing antibodies against IL13. Isotype antibodies were used as control. Media was changed and antibodies added on day 2. 7ADD and annexin V analysis was performed on day 4. As shown in figure 7C IL-13 neutralization resulted in an high mortality of MSC-2 suggesting that indeed the autocrine secretion of this cytokine act as a survival signal for MSC-2. No significant change in MSC-2 viability was observed when isotype control was used. In a parallel plate, MSC-2 were incubated with the anti-IL4Ra aptamer and the pro-apoptotic activity of this molecule was confirmed. In this second plate, media was not changed and the concentration of aptamer used was 10 lower than the antibodies suggesting the superiority of the aptamer in blocking IL-13 signaling.

In the second strategy (fig.7d), $CD11b^+$ cells isolated from 4T1 tumor bearing mice were plated in 24 well plates for 2 days in the presence of IL-13, IFNg or with the combination of the two cytokines. IFNg has been used since we previously shown that the exocrine and autocrine production of this cytokine stimulate the expression of IL4Ra into MDSCs{Gallina, 2006 #10547}. At the end of the culture cells were counted, stained with 7AAD and anti-annexin V antibodies, and analyzed by FACS. As shown in figure 7d only 2x10^5 of the 4x10^5 initially plated were recovered from the wells in which cytokines were not added. Addition of either IFNg or IL13 seems to modestly increment the viability although no statistical significance is reached. When both cytokines are used a significant increment of cells recovered is obtained.

Taken together these data strongly suggest that IL4Ra expression and engagement promote a survival signaling in MDSCs that can be interrupted by the anti-IL4Ra aptamer. Thus, not only we identify a new compound that selectively target MDSCs but, also we, undisclosed for the first time that IL4Ra-STAT6 activation is extremely important for MDSCs survival.

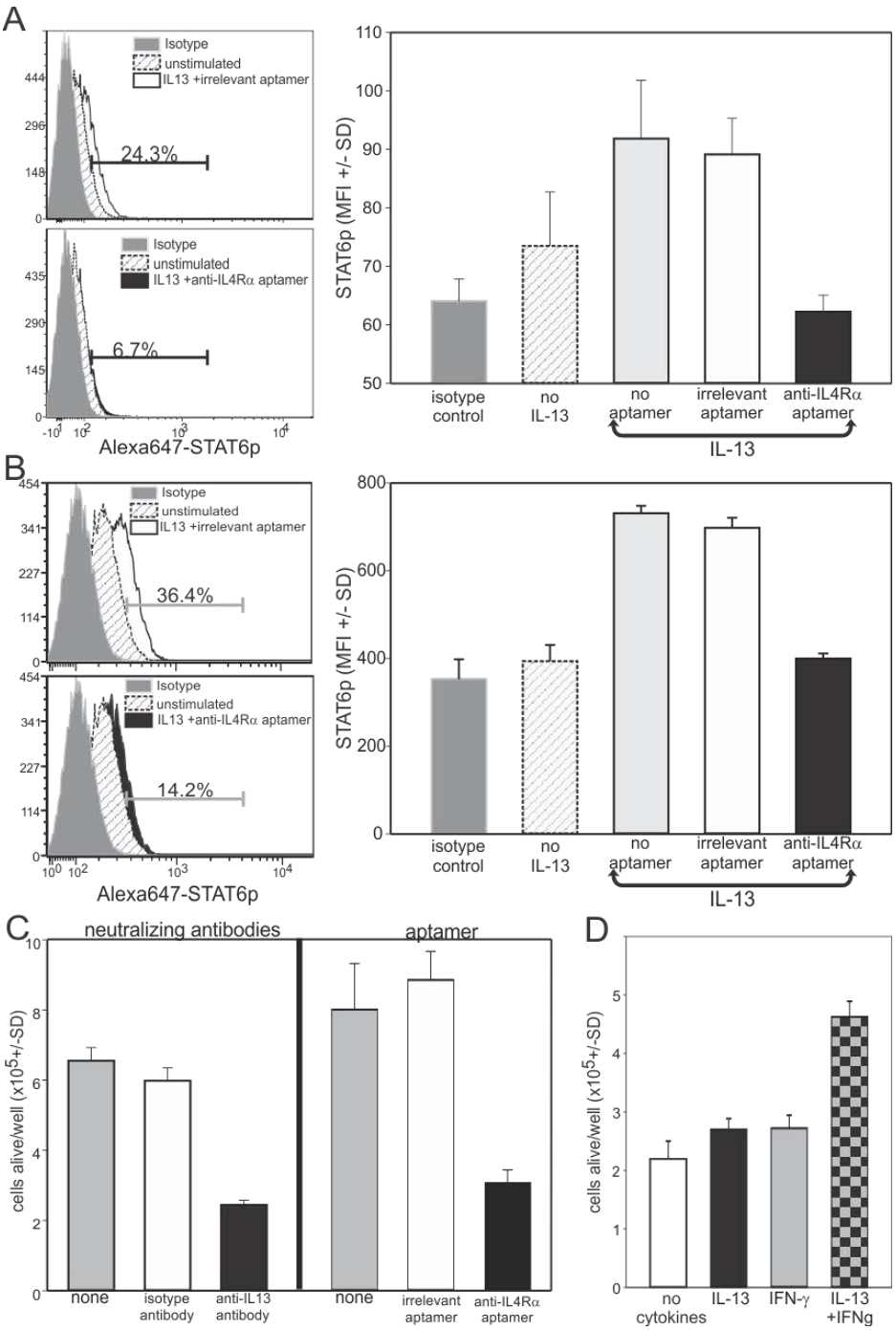

Figure 7: *CD11+ cells isolated from tumor bearing mice (A) or MSC-2 (B) were plated in a 24 well plate. Cells were stimulated with or without IL-13 in the presence of anti-IL4Ra aptamer, of an irrelevant aptamer or no aptamer. Stat6 phosphorylation was evaluated 2 h later by FACs using commercially available kit. C) MSC-2 cells wer incubated for 4 days in the presence or in the absence of anti-IL13 neutralizing antibodies. As comparison anti-IL4Ra aptamer or irrelevant aptamer were used. Alive cells were evaluated by FACS after labeling with 7AAD and anti-annexin V antibodies. D) Splenic CD11b+ cells magnetically purified by tumor bearing mice were cultured in the presence of IFNg, IL-13, both cytokines or none. 24h later cell viability was evaluated by FACS.*

Doxorubicin conjugation to the aptamer.

Although with our surprise the anti-IL4Ra aptamer can significantly reduce tumor progression, it does not result in the complete tumor eradication. As for the initial scope of this study, we wanted to evaluate whether the conjugation of doxorubicin to the aptamer could synergize in promoting tumor regression. Although the conjugation of the doxorubicin to the aptamer was shown to not alter the secondary structure of a PSMA specific aptamer allowing ligand binding, it is important that this is the rule and not the exception. Thus, we first established the optimal condition to bind the doxorubicin to the aptamer using as read out the decreased of the fluorescence spectra observed when doxorubicin intercalates to the nucleic acid{Savla, #15839;Bagalkot, 2006 #15850}. We found that a 20:1 molar doxorubicin-aptamer ratio was optimal for aptamer doxorubicin conjugation (data not shown). A protocol for the purification of these products by selective precipitation has also been established.

In order to verify whether the doxorubicin-aptamer (doxo-aptamer) complex were still able to bind their ligands, anti-IL4Ra doxo-aptamer was incubated with the IL4Ra+ MSC2 cell line. Because of the emission of doxorubicin in the red channel, specific binding could be followed by immune fluorescence microscopy{Savla, #15839;Bagalkot, 2006 #15850}. Briefly, MSC-2 were incubated either with doxorubicin alone or with doxorubicin-aptamer for 2 hours. Cells were than washed and 24h later fluorescence emission was evaluated with a fluorescence microscope. As shown in figure 8, while only few cells are positive when doxorubicin alone is used, almost all the MSC-2 are positive when the anti-IL4Ra doxo-aptamer are used suggesting an active binding of the molecule.

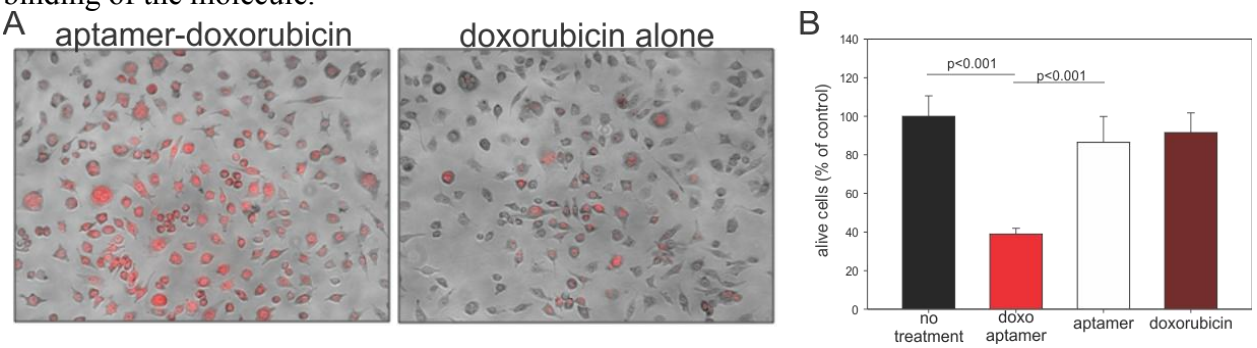

Figure 8: MSC-2 cells were incubated for 2 h with anti-IL4Ra aptamer, anti-IL4Ra aptamer conjugated with doxorubicin, or doxorubicin alone. Cells were washed twice and doxorubicin binding was evaluate by Immune fluorescence microscopy (A). B) MSC-2 treated as in A were cultured for an additional 24h and alive cells determined by trypan blue exclusion assay.

To determine if the higher uptake of doxorubicin correlate with an higher mortality of MDSCs, the experiments was repeated and cell viability evaluated by trypan blue exclusion. As shown in figure 8b, the treatment with either unconjugated aptamer or with doxorubicin alone does not significantly alter MSC-2 viability, most likely because of the short incubation time (24h) for the aptamer or because cells are washed after 2 h removing most of unbound doxorubicin. On the contrary, doxorubicin conjugated aptamer, in the same setting cause the death of more than 60% of the cells. These results, although still preliminary, suggest that doxorubicin can be conjugated to the anti-IL4Ra aptamer without altering its capacity to bind the relevant ligand. Moreover, these results seem to indicate that the conjugation with the aptamer increases the penetrance and specificity of the doxorubicin into the cells. The specificity is further confirmed by parallel experiments in which the 4T1HA IL4Ra negative cell line was used and in which the anti-IL4Ra doxo-aptamer was ineffective.

Conclusion:

In conclusion in the second year of this award we confirm the anti-tumor effect of the anti-IL4Ra aptamer, we understood the mechanism by which this phenomenon happens and we undisclosed a new pathway extremely important for MDSCs survival. These data are currently assembled in a first manuscript. Moreover, we isolate a pool of new aptamers specific for CD11b. Finally we established the condition for the doxorubicin conjugation to the aptamer and determine that these complexes are more efficient than the doxorubicin alone. However, these experiments (doxo-aptamer) need to be confirmed in vivo. The first in vivo experiments with the use of doxorubicin aptamer complex are being performed and the cell selex is proceeding. Because of the additional work required for the functional characterization of the IL4Ra specific aptamer, we were unable to terminate the proposed worked in the two years awarded. For this reason we asked and obtained a 12 month no cost extension and we are confident with this additional time to be able to finish this project.

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Appendices:

None